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Calcineurin Is Required for Sclerotial Development and Pathogenicity of *Sclerotinia sclerotiorum* in an Oxalic Acid–Independent Manner

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Sclerotinia sclerotiorum **is a necrotrophic, omnivorous plant pathogen with worldwide distribution. Sclerotia of *S. sclerotiorum* are pigmented, multihyphal structures that play a central role in the life and infection cycles of this pathogen. Calcineurin, a Ser/Thr phosphatase linked to several signal-transduction pathways, plays a key role in the regulation of cation homeostasis, morphogenesis, cell-wall integrity, and pathogenesis in fungi. We demonstrate that calcineurin expression in *S. sclerotiorum* is altered in a phase-specific manner during sclerotial development. Inhibition of calcineurin by FK506, cysclosporin A, or inducible antisense calcineurin expression impaired sclerotial development at the prematuration phase and increased germination of preformed sclerotia. Induction of antisense calcineurin expression in *S. sclerotiorum* resulted in reduced pathogenesis on tomato and *Arabidopsis*. However, secretion of oxalic acid, a key virulence factor of *S. sclerotiorum,* was not altered. Inhibition of calcineurin conferred a reduction in cell wall β-1,3-glucan content and increased sensitivity to cell-wall-degrading enzymes and to the glucan synthase inhibitor caspofungin. Thus, calcineurin plays a major role in both sclerotial development and pathogenesis of *S. sclerotiorum* and, most likely, other phytopathogens.**

Sclerotinia sclerotiorum is a necrotrophic, phytopathogenic, filamentous ascomycete. It is recognized as an omnivorous plant pathogen with broad host range and worldwide distribution. Over 400 species of plants are susceptible to this pathogen. The majority of these hosts are dicotyledonous, although a number of agriculturally significant monocotyledonous plants also are hosts (Boland and Hall 1994; Purdy 1979; Tu 1997).

The sclerotium of *S. sclerotiorum* is a pigmented, asexual, multicellular, and firm resting structure composed of condensed vegetative hyphal cells which become interwoven and aggregate together, and it is capable of surviving years in soil (Adams and Ayers 1979; Chet and Henis 1975; Tourneau 1979; Willetts and Bullock 1992). Sclerotia germinate to produce saprophytic or infectious mycelia or, carpogenically, to form apothecia. Airborne ascospores derived from the apothecium are the most important means of *S. sclerotiorum* dispersal (Abawi and Grogan 1979; Steadman 1979).

In *S. sclerotiorum*, sclerotial development can be divided into three distinguishable stages (Townsend and Willetts 1954): i) initiation, the appearance of small distinct initial forms of interwoven hyphae, which develop terminally by repeated branching of long, aerial, primary hyphae; ii) development, increase in size; and iii) maturation, characterized by surface delimitation, internal consolidation, and melanization, and often associated with droplet secretion. These phases are accompanied by both morphological and biochemical differentiation. The initiation and maturation stages of sclerotial development are affected by numerous factors, such as photoperiod, temperature, oxygen concentration, mechanical factors, and nutrients (Chet and Henis 1975).

Oxalic acid production has been found to be correlated with sclerotial development and has been shown to be an important factor for pathogenicity in *S. sclerotiorum* and *Botrytis cinerea* (Donaldson et al. 2001; Godoy et al. 1990; Kesarwani et al. 2000; Rollins and Dickman 2001; Zhou and Boland 1999). Enzymes that catabolize oxalate, such as oxalate oxidase or oxalate decarboxylase, protect plants from *S. sclerotiorum* infection in soybean (Donaldson et al. 2001), sunflower (Burke and Rieseberg 2003), and tomato (Kesarwani et al. 2000). Several mechanisms for the involvement of oxalic acid in pathogenesis have been purposed (Bateman and Beer 1965; Cessna et al. 2000; Guimaraes and Stotz 2004; Magro et al. 1984; Maxwell and Lumsden 1970).

In recent years, evidence has accumulated for the involvement of mitogen-activated protein kinases (MAPKs), cAMP, and cAMP-dependent protein kinase A (PKA) in sclerotial development (Chen and Dickman 2005; Chen et al. 2004; Harel et al. 2005; Rollins and Dickman 1998). The reversible nature of protein phosphorylation strongly suggests that protein phosphatases also are associated with this process. One likely candidate for dephosphorylative regulation of sclerotial development is the type 2B protein phosphatase (PP2B; calcineurin).

Calcineurin is a calcium and calmodulin-dependent Ser/Thr protein phosphatase that is conserved in all eukaryotes (Stewart et al. 1982). The core structure of calcineurin is composed of a heterodimer of a catalytic (A) and regulatory (B) subunit. Several regulatory and targeting proteins bind to the core dimer. Calmodulin binds to calcineurin and activates it in the presence of calcium ions. The regulatory (B) subunit of calcineurin is structurally related to calmodulin and possesses four calcium-binding sites that impart calcium-dependent conformational changes to the associated catalytic

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Nucleotide sequence data for *cna1* is available in the GenBank database under accession number DQ182488.

subunit. Upon mobilization of internal calcium stores, the catalytic A subunit is bound by calcium or calmodulin, freeing the active site from the effect of the autoinhibitory domain of the A subunit (Dombradi 1997; Rusnak and Mertz 2000).

Coghlan and associates (1995) demonstrated that, in *Saccharomyces cerevisiae*, a PKA-anchoring protein, AKAP79, binds to calcineurin and PKA simultaneously and inhibits calcineurin. It also has been shown that PKA phosphorylates and negatively regulates the activity of the calcineurin-associated transcription factor Crz1p (Kafadar and Cyert 2004). The functional link between PKA and calcineurin, along with the evidence for the involvement of cAMP signaling in sclerotial development (Chen and Dickman 2005; Chen et al. 2004), support the hypothesis that calcineurin plays a role in sclerotial development.

Calcineurin has been shown to play a central role in the regulation of cation homeostasis, morphogenesis, cell-wall integrity, and pathogenesis in various fungi (Fox and Heitman 2002). Reduction in the expression of the calcineurin catalytic subunit in *Neurospora crassa* was characterized by growth arrest and preceded by an increase in hyphal branching, changes in hyphal morphology, and concomitant loss of the distinctive tip-high, membrane-associated Ca^{2+} gradient typical for growing wild-type hyphae (Prokisch et al. 1997).

Calcineurin also has been shown to play an essential role in the pathogenicity of the human pathogenic fungi *Cryptococcus neoformans* and *Candida albicans*. Calcineurin is required for growth and virulence of *Cryptococcus neoformans* at mammalian body temperature and is required for *Candida albicans* to survive in serum and disseminate in the host (Blankenship et al. 2003; Cruz et al. 2000; Fox et al. 2001; Odom et al. 1997). In the plant pathogen *B. cinerea*, the gene coding for cyclophilin A was identified and inactivated. The null mutant still was able to develop infection structures, but was modified in symptom development on bean and tomato leaves. In addition, inhibition of calcineurin by cyclosporin A (CsA) modified hyphal morphology and prevented infection-structure formation (Viaud et al. 2003). In *Magnaporthe grisea,* mutants of a cyclophilin-encoding gene, *Cyp1*, have been shown to exhibit reduced virulence and are impaired in associated functions, such as penetration-peg formation and appressorium-turgor generation. Furthermore, CsA was found to inhibit appressorium development and hyphal growth. These data implicate cyclophilins as virulence factors in phytopathogenic fungi and provide evidence that calcineurin signaling is required for infection-structure formation by *M. grisea* (Viaud et al. 2002).

Nonetheless, no direct genetic evidence has been produced to date demonstrating the involvement of calcineurin in sclerotial development or its requirement in pathogenesis of phytopathogenic fungi. We demonstrate that *S. sclerotiorum* calcineurin expression is altered in a phase-specific manner during sclerotial development. We report on the inhibition of sclerotial and hyphal development of *S. sclerotiorum* by the specific calcineurin inhibitors FK506 (tacrolimus) and CsA (Jorgensen et al. 2003), and show that calcineurin induces germination of preformed sclerotia. We applied an antisense expression approach to inhibit *cna1* (the *S. sclerotiorum* catalytic subunit calcineurin-encoding gene) expression levels, resulting in the inhibition of sclerotial formation, which was correlated with impairment of the hyphal cell wall. Inhibition of *cna1* expres-

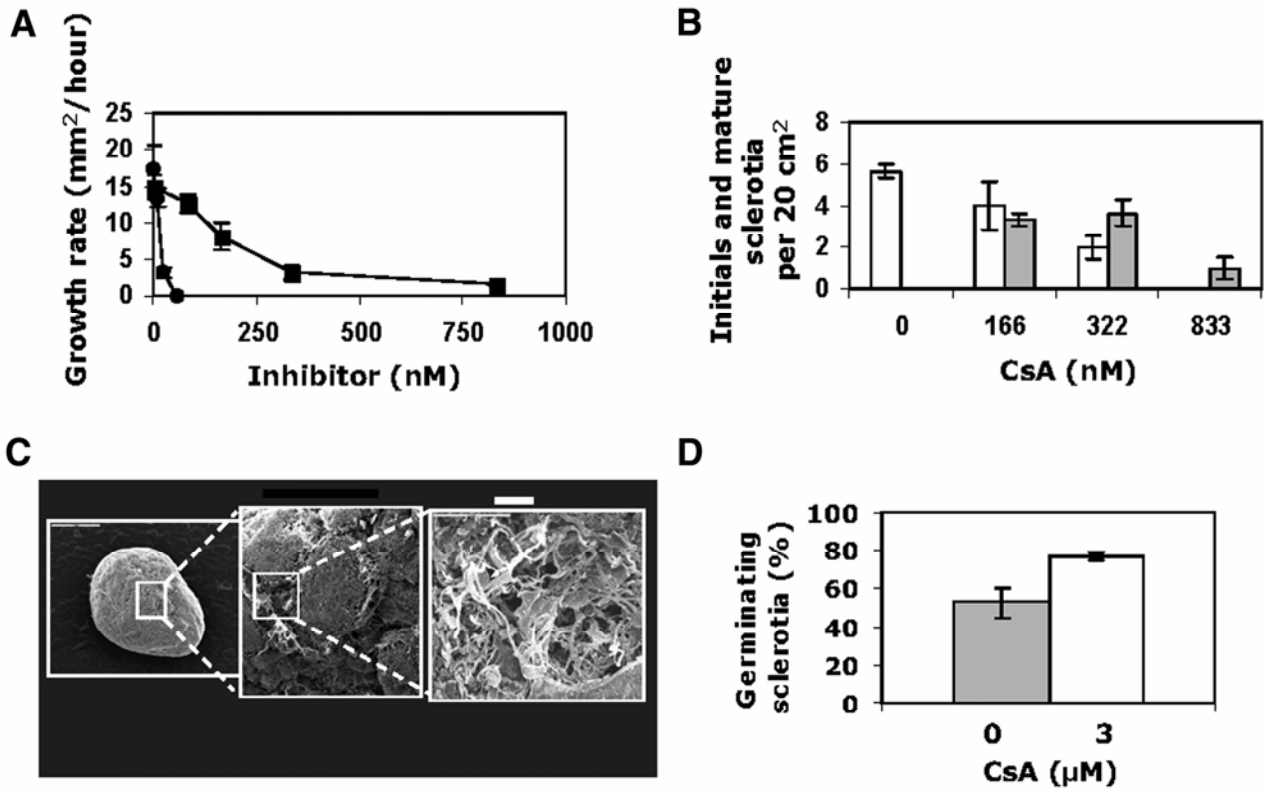

Fig. 1. Effect of cyclosporin A (CsA) and FK506 on sclerotial development, hyphal elongation, and sclerotial germination**. A,** Colony growth rate on solid media supplemented with different concentrations of CsA (squares) or FK506 (circles). In some cases, standard error bars do not exceed symbol borders. **B,** Effect of calcineurin inhibition on initial (gray) and mature (white) sclerotium formation tested under conditions favoring nearly synchronous sclerotial production. Standard error is shown. **C,** Microscopic phase of sclerotial germination (48 h post induction) as visualized by scanning electron microscopy. Bars equal 5 mm (two lower magnifications) and 500 μm (highest magnification). **D,** Sclerotial germination as affected by CsA. Week-old melanized sclerotia were treated with 3 μM CsA, washed, and placed on Joham-bromocresol green medium. Standard error is shown.

sion also was correlated with reduced pathogenicity of *S. sclerotiorum* on *Arabidopsis thaliana* and *Lycopersicon esculentum* and is the first instance in which reduced pathogenicity of *S. sclerotiorum* is not correlated with a reduction in oxalic acid secretion.

RESULTS

FK506 and CsA alter the development of *S. sclerotiorum*.

To determine whether calcineurin activity is involved in the development of *S. sclerotiorum*, we first utilized the structurally unrelated specific inhibitors of calcineurin, CsA and FK506. In the presence of CsA (200 nM) or FK506 (10 nM), hyphal elongation was inhibited by 50% (Fig. 1A). Sclerotial formation was negatively correlated with the concentration of CsA (Fig. 1B) and FK506 (data not shown) in the culture media. At higher inhibitor concentrations (833 nM CsA or 120 nM FK506), sclerotium initials were formed but did not mature to produce sclerotia. Hence, we concluded that the maturation stage of sclerotial development is more sensitive to calcineurin inhibition than the initiation stage (Fig. 1B). Sclerotial germination is characterized first by microscopic hyphal germination (Fig. 1C), followed by the macroscopic presence of hyphal tufts. To analyze the involvement of calcineurin during the early stages (microscopic hyphal germination) of sclerotial germination, we placed CsA-treated sclerotia on medium supplemented with the pH indicator bromocresol green. Pretreating sclerotia with CsA conferred a 25% increase ($P < 0.05$) in sclerotial germination (Fig. 1D). This phenomenon was not observed when sclerotia were pretreated with the protein-synthesis inhibitor hygromycin B (320 μg/ml) or the chitin-synthase inhibitor polyoxin D (5 mM), indicating that the increased germination most likely could be attributed to the specific effect of the calcineurin inhibitor.

Alterations in *cna1* (the *S. sclerotiorum* catalytic subunit calcinerium-encoding gene) expression during sclerotial development.

To determine whether changes in *cna1* expression occurred during sclerotial development, we first cloned the *cna1* gene (discussed below). The *cna1* gene comprises three exons encoding a 516-amino-acid polypeptide which is 77 to 85 and 61% identical to the catalytic subunits of calcineurin in filamentous ascomycetes and human, respectively. We utilized real-time reverse transcriptase-polymerase chain reaction (RT-PCR) to measure *cna1* mRNA abundance in extracts obtained from different morphological stages of asexual development in *S. sclerotiorum* (mature sclerotia, 48 h post germination; hyphae; white sclerotia; and fresh, newly formed mature sclerotia). Differences in *cna1* expression were observed during the different phases of sclerotial development. In contrast to the expression levels detected in the post-germination phase (hyphae), a 2.5-fold increase in expression levels was observed in mature sclerotia and in the newly formed sclerotia (Fig. 2). The decrease in *cna1* transcript levels during germination was correlated with our earlier observation that inhibition of calcineurin increases sclerotial germination by 25% (Fig. 1D). Northern-blot analysis was used to determine *cna1* expression in the presence of CsA. *S. sclerotiorum* was cultured under conditions of nearly synchronous sclerotial formation (Harel et al. 2005) on medium supplemented with 415 nM CsA (IC_{60}) or 833 nM CsA, where the formation of mature sclerotia was totally inhibited. In both instances, a marked increase in the transcript levels of *cna1* was observed in the presence of CsA (Fig. 3). The apparent dose-dependent inhibition effect on *cna1* transcript levels suggests that the abundance of active protein may be involved in regulation of *cna1* transcription.

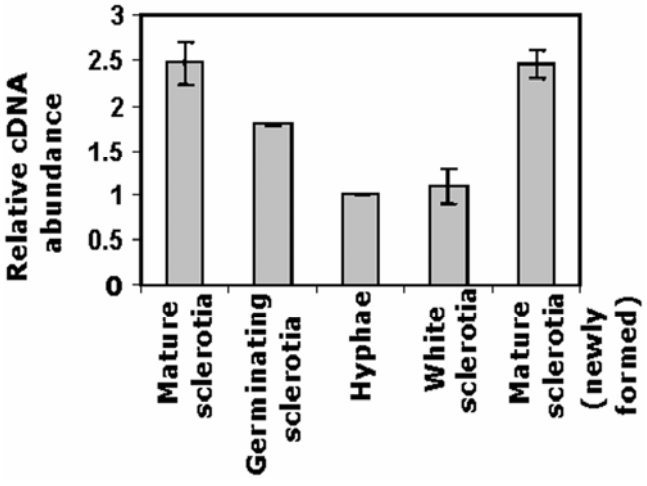

Fig. 2. Alteration in *cna1* expression during sclerotial development as determined by real-time reverse-transcriptase polymerase chain reaction (RT-PCR). The quantity of *cna1* cDNA measured by real-time PCR was normalized to that of β-tubulin cDNA within each reaction. "Newly formed sclerotia" are those formed from growing hyphae during the time course of the experiment. Data shown are the average of the experiments. Standard error is shown. The relative abundance of cDNA from the hyphal samples was arbitrarily assigned a value of 1.

Genetic inhibition of calcineurin attenuates sclerotial development in *S. sclerotiorum*.

The results demonstrating inhibition of hyphal growth and sclerotial production by calcineurin-specific inhibitors, coupled with the alteration in *cna1* expression levels during sclerotial production, suggest that *cna1* plays an important role in sclerotial development. Because disruption mutations in the *Aspergillus nidulans cna* encoding gene are lethal (Rasmussen et al. 1994), we anticipated that the same would apply for the *cna1* gene of *S. sclerotiorum*. Therefore, we chose to test the hypothesis that *cna1* plays an important role in sclerotial development by inhibition of the target function via the regulated expression of *cna1* antisense RNA in transgenic *S. sclerotiorum* strains (i.e., to create conditional mutations in which *cna1* expression levels and calcineurin activity are reduced via an inducible *cna1*-antisense cassette).

We cloned the *S. sclerotiorum cna1* gene (see Experimental Procedures). An approximately 1.1-kb cDNA fragment of *cna1* was inserted into the pSO-1 vector in antisense orientation,

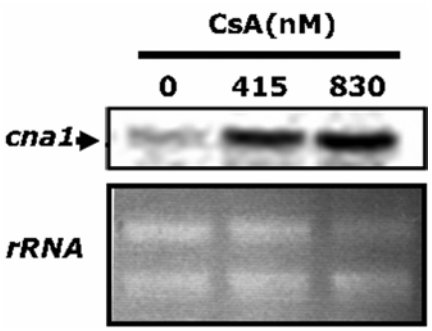

Fig. 3. Effect of cyclosporin A (CsA) on *cna1* transcript levels. Northern blot analysis of *cna1* transcript in total RNA extracted from *Sclerotinia sclerotiorum* hyphae grown under conditions of near-synchronous sclerotial growth, in medium supplemented with CsA (415 nM, 60% inhibition; 833 nM, 100% inhibition). General RNA abundance was visualized by direct ethidium bromide staining (lower panel).

downstream of the *N. crassa* quinic acid 2 gene promoter (Fig. 4A). The gene *qa-2* encodes for the *N. crassa* catbolic hydroquinase (EC 4.2.1.10), which is part of a gene cluster involved in uptake and catabolism of quinate. The fact that the *qa-2* promoter is quinic acid-inducible has made it a convenient tool for regulating homologous and heterologous transgene expression in fungi (Chen et al. 2004; Perkins et al. 2001). The resulting construct, pSB2, was used to transform the wild type of *S. sclerotiorum*, and pSB2-containing transformants (verified by Southern analysis) were isolated. When the expression of *cna1* in antisense orientation was induced by quinic acid (15 mM), *cna1* transcript levels were markedly reduced (Fig. 4B). Interestingly, the abundance of *cna1* transcript in the glucose-cultured CAS1 strain appears to be somewhat higher than in the wild type. A possible explanation for this observation is that even minor activity of the *qa-2* promoter in the presence of glucose results in slight *cna1* antisense production, a subsequent reduction in calcineurin activity, and activation of *cna1* transcription (as observed in the case of CsA amendment) (Fig. 3).

Total calcineurin phosphatase activity was measured in an *S. sclerotiorum cna1* antisense transformant (designated CAS1) grown on antisense-induced (quinic acid) or repressed (glucose) media. An approximate 50% reduction in relative calcineurin activity was measured in the CAS1 strain grown in the presence of quinic acid. The presence of quinic acid in the media of the wild-type control strain did not reduce calcineurin activity, indicating that inhibition of calcineurin activity was not affected by the quinic acid supplement (Fig. 4C).

When the CAS1 (as well as other pSB-2 transformants) strain was cultured on solid media supplemented with quinic acid, where *cna1* expression levels were shown to be reduced, sclerotial formation was attenuated significantly (Fig. 4D), as observed by a 72-h delay in the appearance of sclerotia (in liquid culture, even hyphae developed poorly). These results confirmed our hypothesis that calcineurin plays a role in sclerotial development. We chose the CAS1 strain for further experimentation. Furthermore, when sclerotia produced by the CAS1 strain (grown in the presence of glucose) were pretreated with quinic acid prior to the germination assay, we observed a 20%

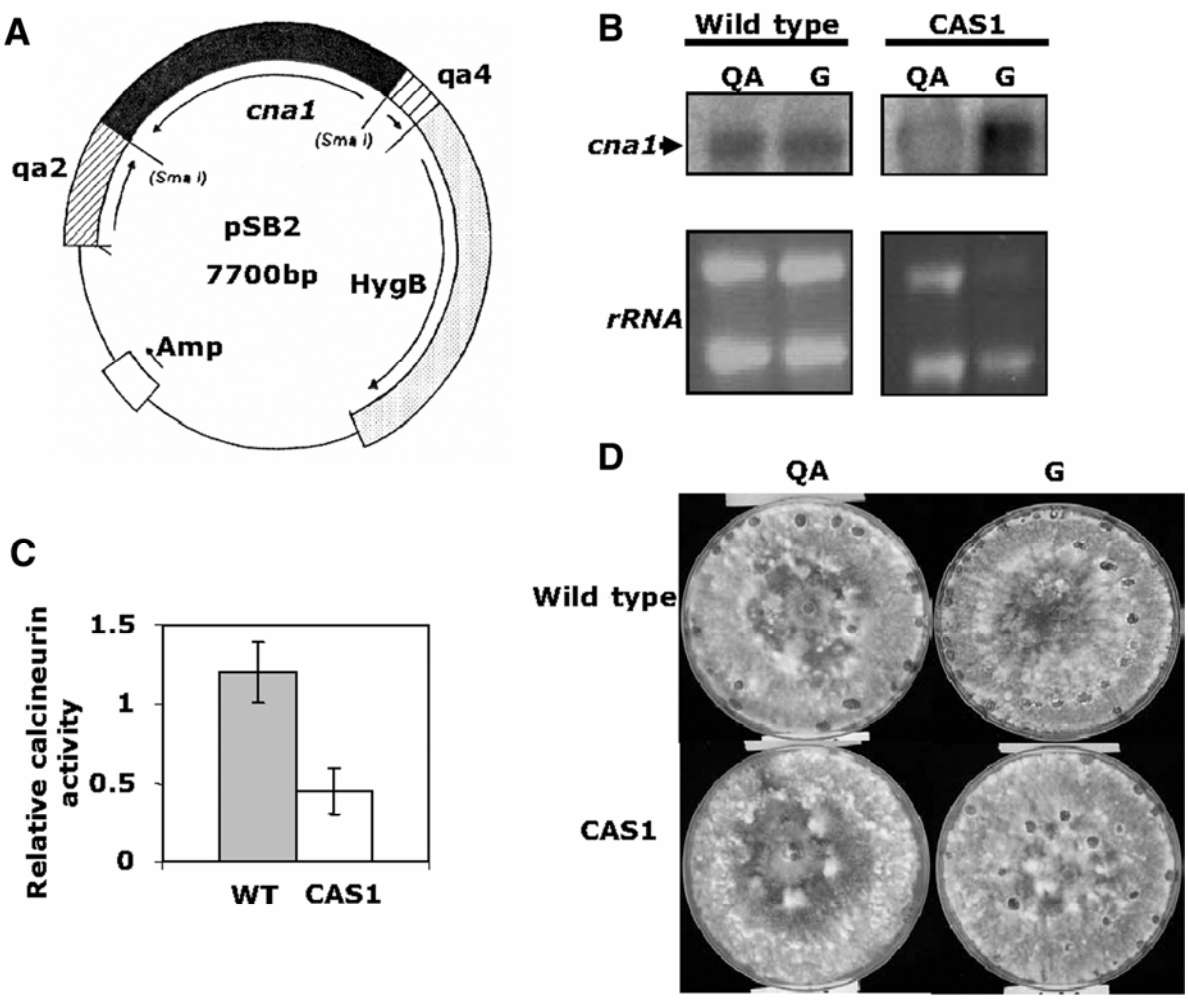

Fig. 4. Construction of a *cna1*-antisense RNA expression vector, pSB2, and functional analysis of the calcineurin antisense strain (CAS1). **A,** A 1.1-kb fragment of *cna1* was inserted in antisense orientation between the promoter region of the *Neurospora crassa* qa-2 gene and a 380-bp fragment which includes regions containing polyadenylation and termination signals from the qa-4 gene. **B,** Expression of *cna1* (arrow) in the *Sclerotinia sclerotiorum* CAS1 strain grown on qa-2-inducing (QA; 15 mM quinic acid) or repressing (G, 15 mM glucose) medium as determined by Northern-blot analysis. Equal loading of RNA was confirmed by direct ethidium bromide staining (lower panel). **C,** Relative specific activities of calcineurin (ratio of antisense-induced to antisense-repressed) in extracts of the *S. sclerotiorum cna1*-antisense transformants (CAS1) or the wild-type strain. Calcineurin activity was determined by measuring the dephosphorylation rate of the RII synthetic phosphopeptide substrate. Standard error is shown (*t* test, $P < 0.05$). **D,** Phenotype of an *S. sclerotiorum cna1*-antisense transformant. Wild-type isolate 1980 (top) and the CAS1 strain (bottom) were grown in medium supplemented with 15 mM glucose (G), where antisense expression is repressed, or 15 mM quinic acid (QA), where antisense expression is induced. The cultures were photographed 10 days post inoculation.

increase in sclerotial germination relative to sclerotia pretreated with glucose. These results are well correlated with our earlier observation that pretreatment of wild-type sclerotia with CsA increases sclerotial germination (Fig. 1D) and demonstrate that the involvement of calcineurin in sclerotial development of *S. sclerotiorum* is phase dependent.

The *cna1* antisense strain of *S. sclerotiorum* exhibits reduced fungal pathogenesis.

Calcineurin-associated proteins have been shown to be involved in the pathogenesis process of *B. cinerea* and *M. grisea*, although no genetic evidence for the involvement of calcineurin in the pathogenesis process of phytopathogenic fungi has been presented to date (Viaud et al. 2002, 2003). To determine whether a reduction in calcineurin activity would confer a change in the pathogenic capability of *S. sclerotiorum*, we inoculated detached tomato leaves with agar plugs colonized with the CAS1 strain grown in the presence of either quinic acid or glucose. Inhibition of *cna1* transcript levels conferred a significant ($P < 0.05$) reduction (of approximately 80%) in fungal virulence (Fig. 5A) as measured 96 h post inoculation by monitoring the number of leaves in which necrotic lesions appeared in proximity to the fungal colony. *S. sclerotiorum* is a broad-spectrum pathogen; therefore, we expanded the pathogenicity assay to an additional host: *Arabidopsis thaliana*. In this case as well, inhibition of *cna1* expression levels conferred a significant ($P < 0.05$) reduction (approximately 80%) in the virulence of *S. sclerotiorum* on the cruciferous host (Fig. 5B), indicating that this phenomenon is not restricted to the interaction between *S. sclerotiorum* and tomato.

Oxalic acid secretion is not impaired during inhibition of *cna1* transcript levels.

Oxalic acid has been shown to play a role in the pathogenesis process of *S. sclerotiorum*; therefore, we analyzed the production of oxalic acid under *cna1*-inhibiting conditions, the CAS1 strain, the wild-type strain, and the A-1 mutant (which does not produce sclerotia, does not secrete oxalic acid, and is not pathogenic) in the presence of quinic acid or glucose. Cultures were harvested after hyphal growth reached a diameter of 8 cm and the accumulation of oxalic acid in the medium was measured. The presence of quinic acid (15 mM) in the culture media did not significantly affect oxalic acid secretion by the wild-type or CAS1 strain (Fig. 6). Thus, the presence of quinic acid in the culture media attenuated sclerotial formation and reduced pathogenicity of the CAS1 strain by 80% without any concomitant effect on oxalic acid secretion.

Because the secretion of oxalic acid, a major pathogenicity determinant, is not impaired in the CAS1 strain grown under *cna1*-inhibiting conditions, additional experiments were performed to determine whether a change in the secretion of alternative virulence factors, such as total proteases, cellulases, or pectinases, occurs in the CAS1 strain. Based on our results, no significant change in the secretion of any of these enzymes had occurred in the CAS1 strain (data not shown), suggesting that at least some of the *S. sclerotiorum* virulence determinants apparently are unaffected by the reduction in calcineurin activity.

Biochemical and genetic inhibition of calcineurin impairs the integrity of the fungal cell wall.

Calcineurin is known to play a role in the regulation of cell-wall biosynthesis in *S. cerevisiae*. It acts as a positive regulator of the expression of FKS2, encoding a component of the β-1,3-glucan synthase complex necessary for cell-wall integrity (Zhao et al. 1998). To analyze the possible involvement of calcineurin in β-1,3-glucan synthesis in *S. sclerotiorum*, we investigated whether antisense-based inhibition of calcineurin would render the strain hypersensitive to the echinocandin drug caspofungin, which is a known inhibitor of β-1,3-glucan synthase (Kurtz et al. 1996). Petri dishes, containing minimal media supplemented with caspofungin at 0.015 μg/liter (a concentration that inhibited 50% of radial hyphal growth), were inoculated with the CAS1 strain grown in the presence of either quinic acid or glucose. Based on repeated experiments, hyphae of the CAS1 strain grown in the presence of both quinic acid and caspofungin were inhibited over 30% more than the CAS1 strain grown in the presence of caspofungin alone, whereas radial growth of CAS1 strains grown on quinic acid was not

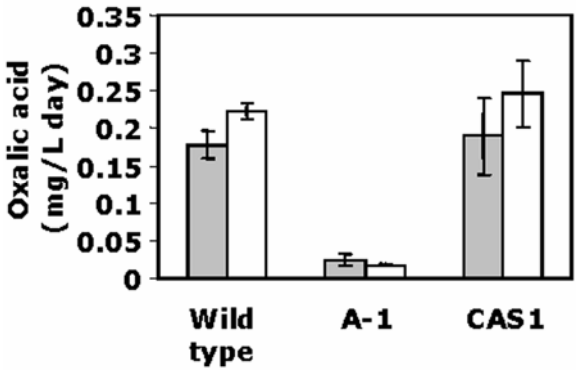

Fig. 6. Oxalic acid secretion by three *Sclerotinia sclerotiorum* strains. Oxalic acid accumulation in potato dextrose agar medium of wild-type, A-1, and CAS1 strains grown in the presence of 15 mM glucose (gray, antisense repressor) or 15 mM quinic acid (white, antisense inducer). Standard error is shown.

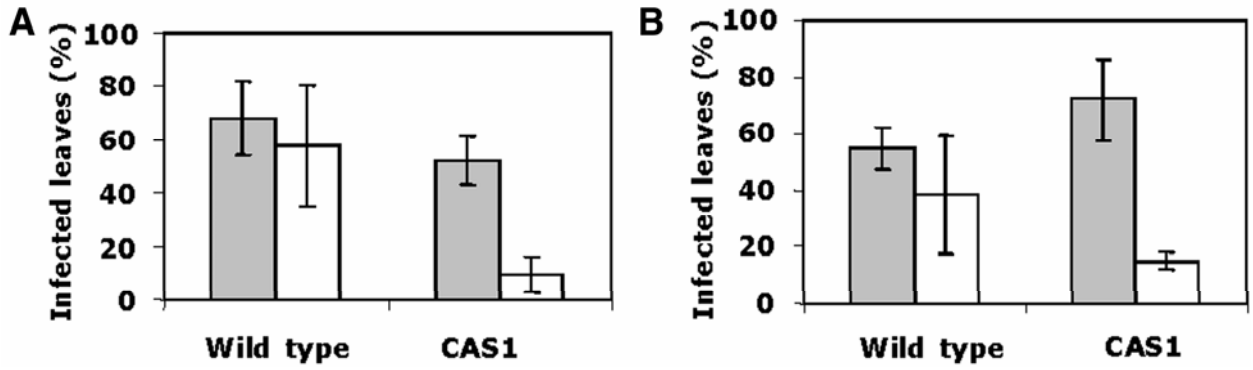

Fig. 5. Effect of *cna1* inhibition on *Sclerotinia sclerotiorum* pathogenicity. **A,** Tomato (cv. Bonny Best) or **B,** *Arabidopsis thaliana* accession Co-0 leaves were inoculated with water-agar plugs supplemented with 15 mM glucose (gray) or 15 mM quinic acid (white), colonized with the wild-type or *cna1*-antisense transformant (CAS1), and incubated for 96 h. Standard error is shown.

inhibited (Fig. 7A). Therefore, we concluded that inhibition of calcineurin and β-1,3-glucan synthase confers a synergistic effect on hyphal growth of *S. sclerotiorum*. To further inquire whether inhibition of calcineurin could reduce the synthesis of β-1,3-glucan in *S. sclerotiorum*, we evaluated the β-1,3-glucan content in hyphae grown in either the presence or absence of CsA. When the wild-type strain was grown in potato dextrose broth (PDB) (the CAS1 mutants do not grow well in liquid culture) supplemented with 50 nM CsA, the amount of cell-wall β-1,3-glucan was reduced by approximately 40% compared with the untreated control ($P < 0.05$) (Fig. 7B).

Finally, we tested whether the reduction in β-1,3-glucan content was correlated with hypersensitivity to cell-wall-degrading enzymes. Protoplasts were produced from hyphae grown in either the presence or absence of CsA. The number of protoplasts produced from the wild-type strain grown in PDB medium supplemented with 50 nM CsA during the 60-min experiment was approximately twofold higher than the number of those produced from the wild type grown in the control medium (Fig. 7C), indicating that, under these conditions, the fungus is more susceptible to cell-wall-degrading enzymes.

Studies of the infection process of *S. sclerotiorum* on bean hypocotyls and pea pods have documented the formation of infection cushion structures which may participate in the penetration and pathogenicity process (Huang and Kokko 1992; Lumsden and Dow 1973). The observed reduction in β-1,3-glucan content and the apparent sensitivity of the hyphal cell wall to cell-wall-degrading enzymes in hyphae grown in the presence of the calcineurin inhibitor also could result in abnormal infection cushion formation (and subsequent reduced pathogenicity). To determine whether a reduction in calcineurin activity would confer a change in the production of infection cushions, we placed agar plugs colonized with the CAS1 strain grown in the presence of either quinic acid or glucose on a transparent hydrophobic surface (empty petri dishes), conditions inductive for infection cushion formation. Under all conditions tested, no alteration in infection cushion production rate or morphology were observed (Fig. 7D). Thus, inhibition of calcineurin conferred a reduction in pathogenicity and impaired cell wall integrity but did not affect the formation and the morphology of these infection-associated structures.

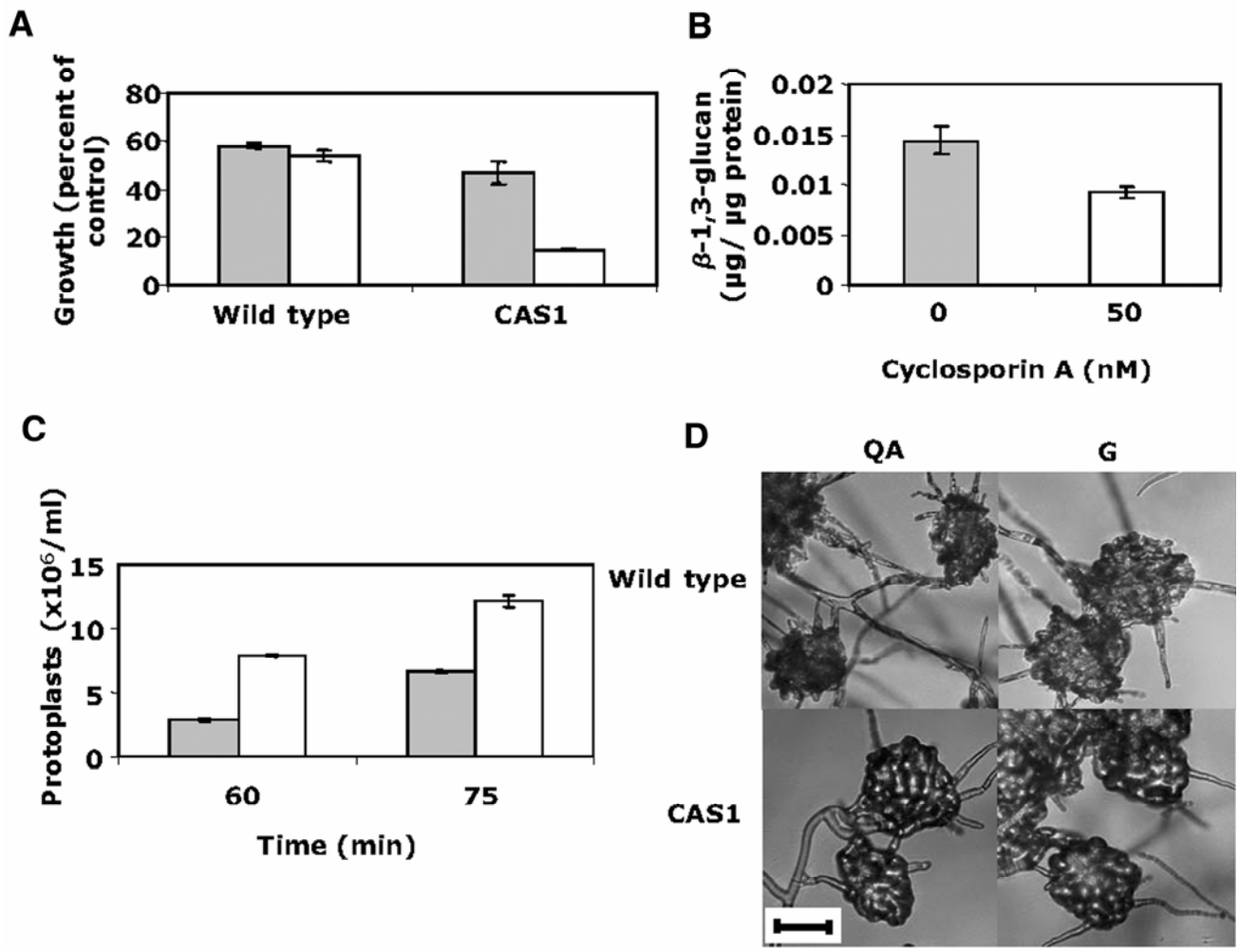

Fig. 7. Effect of calcineurin inhibition on cell wall and infection cushion formation. **A,** Effect of antisense-based inhibition of calcineurin on hyphal growth in the presence of caspofungin. Petri dishes containing minimal media supplemented in the presence or absence of caspofungin at 0.015 µg/liter caspofungin were inoculated with the CAS1 or the wild-type strains grown in the presence of either 15 mM glucose (gray) or 15 mM quinic acid (white). Results represent the percent growth of that on control medium in the absence of caspofungin. Standard error is shown. **B,** Effect of cyclosporin A (CsA) on accumulation of β-1,3-glucan in the fungal cell wall. β-1,3-Glucan content was analyzed in hyphae of the wild-type strain grown in either the presence (white) or absence (gray) of 50 nM CsA. Standard error is shown. **C,** Protoplast production in wild-type *S. sclerotiorum*, as affected by the presence (white) or absence (gray) of 50 nM CsA. Standard error is shown. **D,** Effect of antisense-based inhibition of calcineurin on infection cushion formation. Potato dextrose agar plugs supplemented with 15 mM glucose (G) or 15 mM quinic acid (QA), colonized with the wild-type (top) or *cna1*-antisense transformant (CAS1; bottom) 24 h after transfer to a hydrophobic surface. Bar indicates 50 µM.

DISCUSSION

Even though sclerotium-producing fungi belong to various different phyla of the fungal kingdom, analysis of the molecular mechanisms governing sclerotial formation, maintenance, and germination is still in its infancy. The involvement of Ser/Thr protein kinases in sclerotial formation has been demonstrated. However, to date, the roles of protein phosphatases in this developmental process have not been probed.

Calcineurin plays an essential role in the development and pathogenesis of *C. albicans* and *Cryptococcus neoformans*, two medically important fungal pathogens (Fox and Heitman 2002; Kraus et al. 2005). However, among phytopathogenic fungi, the involvement of calcineurin in the pathogenesis process has been demonstrated only by utilizing specific calcineurin inhibitors or, indirectly, by impairing the genes encoding cyclophilin A (Viaud et al. 2002, 2003). The goal of this research was to explore the requirement of calcineurin for sclerotial development and pathogenicity of *S. sclerotiorum*.

We established four lines of evidence for the involvement of calcineurin in sclerotial formation: i) specific biochemical inhibitors of calcineurin (FK506 and CsA) inhibited sclerotial formation, mainly at the maturation stage; ii) the clear changes in *cna1* expression levels that accompany distinct phases of sclerotial formation and germination imply that these changes are part of the developmental process; iii) inhibition of calcineurin by CsA invoked an increase in *cna1* expression levels, suggesting the significance of maintaining sufficient levels of calcineurin activity during sclerotial development; and iv) antisense expression-based inhibition of *cna1* transcript levels attenuated the rate of sclerotial development.

In addition, both pharmacological- and genetic-based inhibition of calcineurin (utilizing CsA and *cna1*-antisense, respectively) increased sclerotial germination, indicating that calcineurin involvement in the multistage process of sclerotial morphogenesis is dependent on the specific phase of fungal-structure development. Furthermore, the fact that reduction of calcineurin levels increased sclerotial germination implies that, at a certain stage of sclerotial development, it is conceivable that calcineurin may be involved in natural inhibition of sclerotial germination. Whether or not these effects occur in other fungal resting-propagule structures has yet to be determined. However, these findings may have implications for the potential detrimental effects (when curbing fungal growth is desired) of applying a sublethal dose of inhibitor when plant or human pathogens are involved.

Calcineurin has been shown to be involved in the development of different morphological stages requiring de novo cell-wall biosynthesis in various fungi (Cruz et al. 2001; Mendoza et al. 1996; Prokisch et al. 1997; Steinbach et al. 2004; Viaud et al. 2002, 2003; Yoshida et al. 1994). However, the function of calcineurin in sclerotial development in fungi has not been demonstrated. The fact that the sclerotia are composed of dense cell-wall material may reflect the significant contribution calcineurin has to sclerotial formation and maturation.

Calcineurin involvement in the pathogenesis process of the mammalian pathogens *Candida albicans* and *Cryptococcus neoformans* has been demonstrated clearly. Even though previous studies have shown that calcineurin-associated proteins are required for pathogenicity of the phytopathogenic fungi *B. cinerea* and *M. grisea* (Viaud et al. 2002, 2003), this is the first instance exhibiting the requirement for calcineurin on the basis of genetic manipulation. We show that calcineurin is involved in the pathogenesis process of *S. sclerotiorum* on two diverse hosts, tomato and *A. thaliana.* In both host–pathogen systems, antisense-based inhibition of *cna1* significantly decreased (by 80%) the ability of *S. sclerotiorum* to infect plant leaves.

Oxalic acid and lytic enzymes have been suggested to play significant roles in the pathogenesis of *S. sclerotiorum* on its hosts (Cotton et al. 2003; Favaron et al. 2004); therefore, we tested whether antisense-based inhibition of calcineurin impairs the secretion of oxalic acid and extracellular lytic enzymes. No reduction in oxalic acid secretion by the CAS1 strain or the wild type was found under the conditions we tested and the level of oxalic acid secreted into the medium (0.2 mg/ml/day) was similar to those previously described by Rollins and Dickman (1998). Thus, it is reasonable to postulate that calcineurin is required for pathogenesis in an oxalic acid-independent manner. This is a unique observation, because oxalic acid seems to be a pivotal player in the pathogenic mechanism of *S. sclerotiorum* (Burke and Rieseberg 2003; Cessna et al. 2000; Donaldson et al. 2001; Guimaraes and Stotz 2004; Magro et al. 1984; Maxwell and Lumsden 1970; Zhou and Boland 1999) and, so far, all reports on strains of *S. sclerotiorum* exhibiting reduced pathogenicity, in which oxalic acid secretion was monitored, have stated that a reduction in oxalic acid secretion (in vitro, in planta, or both) accompanies the reduced pathogenic phenotype. Even though no changes in oxalic acid secretion were observed in culture during the course of this study, the possibility that such a reduction occurs in planta, during the fungus–plant interaction, has yet to be analyzed. In addition to the lack of change in oxalic acid secretion, we could not detect any notable reduction in the secretion levels of additional extracellular components (protease, cellulase, or pectinase) known to be involved in the necrotrophic lifestyle of this pathogen when the CAS1 strain was grown under antisense-inducible conditions.

Phytopathogenic fungi subjected to calcineurin- or cyclophilin-inhibiting conditions exhibit defects in morphological structures (such as appressoria and penetration pegs) which are essential for pathogenesis (Viaud et al. 2002, 2003). The attenuation of sclerotial development and hyphal growth in *S. sclerotiorum* following genetic and biochemical inhibition of calcineurin could well be accompanied by defects in additional morphological features that are important for pathogenicity. One of these may be the production of infection cushions, even though, at least in some instances, they were shown to be not essential for pathogenicity (Huang et al. 1997; Jamaux et al. 1995). In this study, we did not detect any inhibition or morphological changes in the infection cushions when the CAS1 strain was grown under antisense-inducible conditions.

Mutations in the calcineurin catalytic subunit of *Candida albicans* confer hypersensitivity to cell-wall-perturbing agents, such as sodium dodecyl sulfate (SDS), calcofluor white, and congo red (Sanglard et al. 2003). In addition, a functional link between calcineurin and glucan synthesis has been established in *S. cerevisiae* (Stathopoulos and Cyert 1997; Zhao et al. 1998). Therefore, we tried to determine whether impairment in *S. sclerotiorum* cell-wall structure occurs in hyphae grown under calcineurin-inhibiting conditions. The fact that CAS1 strains exhibited elevated sensitivity to the specific β-1,3-glucan synthase inhibitor caspofungin, and that hyphal cells of the wild type that were grown under calcineurin-inhibiting conditions exhibited a reduction in β-1,3-glucan content and were more susceptible to cell-wall-degrading enzymes (as expressed by the accelerated production of protoplasts from hyphae grown in the presence of CsA), is indicative of significant defects in the cell wall in general and the glucan component in particular. Furthermore, because the role of glucans in fungal pathogenesis has been demonstrated in the past (Herrero et al. 2004; Kapteyn et al. 2000; Rappleye et al. 2004; Staab et al. 1999), it is possible that the observed reduction in β-1,3-glu-

can content of hyphae in which calcineurin activity was inhibited also could contribute to the observed reduction in pathogenesis of *S. sclerotiorum*. Our findings concerning the plant pathogen are supported by previous clinical findings with the human pathogens *Aspergillus fumigatus* and *Cryptococcus neoformans* describing the clear synergistic interactions between FK506 or CsA and caspofungin (Del Poeta et al. 2000; Steinbach et al. 2004).

Sclerotial development is a complex, multistage process which is thought to be regulated by signal-transduction pathways such as MAPK and PKA (Chen and Dickman 2005; Chen et al. 2004; Harel et al. 2005; Rollins and Dickman 1998). Recently, evidence has been produced for the existence of calcineurin-MAPK- and calcineurin-PKA-associated pathways. For example, in *S. cerevisiae*, PKA has been shown to phosphorylate and, consequently, negatively regulate the activity of the calcineurin-regulated Zn-finger transcription factor Crz1p (Kafadar and Cyert 2004) by inhibiting its nuclear import (Kafadar and Cyert 2004). In human cells, transcriptional activity of NFATc2 (a Crz1p homolog) is upregulated by phosphorylation of the MAPK JNK (Ortega-Perez et al. 2005). If similar pathways exist in *S. sclerotiorum*, our current analysis demonstrating that calcineurin plays a significant role in the regulation of morphogenesis and pathogenesis in this pathogen may serve as a basis for the further dissection of these pathways. Understanding of the physiological and molecular mechanisms involved in sclerotial development and pathogenicity of *S. sclerotiorum* may well reflect the development and pathogenesis of additional sclerotium-producing fungi and may provide new avenues for intervention in these processes, leading to improved control of disease caused by sclerotium-borne fungi.

MATERIALS AND METHODS

S. sclerotiorum growth conditions.

Wild-type *S. sclerotiorum* isolate 1980 and the mutant A-1, deficient in oxalic acid production and sclerotial formation (Godoy et al. 1990), were used in this study. The *cna1* antisense strain (designated CAS1) harbors a construct expressing the *S. sclerotiorum* catalytic subunit of the calcineurin gene (*cna1*) in antisense orientation. Strains were routinely cultured, unless otherwise stated, on potato dextrose agar (PDA; Difco Laboratories, Detroit). When required, the growth medium was supplemented with hygromycin B at 66 μg/ml (Calbiochem, Riverside, CA, U.S.A.). When measuring the effect of FK506 (Calbiochem) and CsA (Calbiochem) on *S. sclerotiorum* development, the fungus was grown on Joham's defined medium (Harel et al. 2005).

To determine the interaction between caspofungin and genetic-based inhibition of calcineurin, agar plugs cultured with the CAS1 or wild-type strains were used to inoculate dishes containing water agar media supplemented, or not, with caspofungin at 0.015 μg/liter (Merck, Rahway, NJ, U.S.A.) in the presence of 15 mM quinic acid or 15 mM glucose, which then were incubated at 18°C for 5 to 6 days. The experiment was repeated twice, in triplicate, and the average result of the two experiments is presented.

Where inhibitors were used, appropriate controls with the relevant solvents were performed (ethanol for CsA and methanol for FK506).

The effect of FK506 and CsA on sclerotial formation and hyphal growth.

To analyze the effect of FK506 and CsA (Calbiochem) on hyphal elongation, agar plugs cultured with the wild-type strain were used to inoculate dishes containing medium supplemented with different concentrations of the calcineurin inhibitors. Each experiment was performed at least three times in three repeats for each concentration. Results indicate the average colony area (estimated by measuring the diameter of the fungal colony) and the variation among three replicates of a selected experiment.

To test the effect of FK506 and CsA (Calbiochem) on sclerotial development, *S. sclerotiorum* was cultured under conditions of nearly synchronous sclerotial formation (Harel et al. 2005), on medium supplemented with different concentrations of CsA or FK506 (Calbiochem). Each experiment was performed three times in three repeats for each concentration. Results indicated the average number of sclerotia and sclerotial initials and the variation among three technical replicates of a selected experiment.

Sclerotial germination tests.

Week-old sclerotia were submerged in 10% sodium hypochlorite for 1 min and washed three times with sterile distilled water. For treatment with CsA, the sclerotia were submerged in 3 μM drug for 15 min, washed again (twice) with sterile distilled water, and placed over Joham-bromocresol green medium. This medium was prepared by overlaying solid Joham medium with 0.1% (wt/vol) bromocresol green (Sigma-Aldrich, St. Louis) solution for 5 min, followed by discarding of the bromocresol green solution. Germination experiments were repeated three times and results were subjected to *t* test analysis. As sclerotia germinate, they secrete oxalic acid, which changes the color of the pH indicator to yellow, enabling early scoring of sclerotial germination (Godoy et al. 1990) followed by the production of mycelial tufts on the germinating sclerotia.

Scanning electron microscopy.

For scanning electron microscopy (SEM), dormant or germinating (48 h) sclerotia were fixed for 4 h with 5% (vol/vol) glutaraldehyde in 0.1 M phosphate buffer, pH 7.2. The samples were washed five times with the same buffer and then dehydrated in a series of 25 to 100% ethanol washes (Yatzkan and Yarden 1999). The fixed samples were dried for 1 h in a CPD750 drier (Bio-Rad, Hercules, CA, U.S.A.) and gold-coated in a E5150 Polaron SEM coating system apparatus (Bio-Rad). The samples were observed under a Jeol (Tokyo, Japan) JSM 35 microscope.

Nucleic acid isolation and manipulation.

Standard recombinant DNA methods were performed according to (Sambrook et al. 1989). PCR was performed using SuperTerm JMR801 Polymerase (MBI Fermentas, Vilnius, Lithuania). RNA extraction was performed by quick-freezing samples in liquid nitrogen followed by grinding the samples by mortar and pestle. Total RNA was extracted with TRI reagent (Sigma-Aldrich). When needed, mRNA was isolated from total RNA samples using the PolyA-Tract kit system (Promega Corp., Madison, WI, U.S.A.). mRNA was eluted according to the manufacturer's instructions with 250 μl of nuclease-free water and samples were stored at –80ºC until use.

Northern-blot analysis was performed according to standard procedures (Sambrook et al. 1989). Total RNA samples (20 μg/lane) were transferred to Magnacharge NT nylon membranes (MSI, Westborough, MA, U.S.A.). The blots were probed with an [α-^{32}P] dCTP hexamer-labeled DNA probe (Prime-A-Gene; Promega Corp.) prepared from the 1.1-kb fragment of *cna1* (see below) as template. Hybridization was performed at 42°C in the presence of ULTRAhyb solution (Ambion, Austin, TX, U.S.A.). The most stringent washes were carried out at 42°C with 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 0.1% (wt/vol) SDS.

Production of *cna1* antisense construct.

The *S. sclerotiorum* gene encoding the catalytic subunit of calcineurin (designated *cna1*) was cloned by initially amplifying a 368-bp fragment of the *cna1* gene using the following degenerate primers, primarily designed for cloning Ser/Thr protein phosphatases: degF-(GATGAATTCGGNGAYTAYGT NGAYMGNGC) and degR-(CGTGGATCCRTCNGGATCNG ACCANARNARRTC). The 368-bp fragment of *cna1* was further used, as a probe, to detect a cosmid harboring the *cna1* gene in an *S. sclerotiorum* genomic library (Rollins and Dickman 2001), from which the gene was further sequenced (GenBank accession number DQ182488).

An approximately 1.1-kb cDNA fragment of the coding region of *cna1* first was amplified by PCR with the primers *cna1*F-(GCTATGGTTCCACCGACAGA) and *cna1*R- (ATG GGACCTGGAGAAGTCCT) and cloned into a pDrive vector (Qiagen, Hilden, Germany). The unique *Sna*bI (pDrive) and *Hinc*II (*cna1*) sites were digested and the excised fragment was ligated into pSO-1 (linearized by *Sma*I) to produce pSB2 (Fig. 4A). pSO-1 was constructed on the basis of a vector described by Fecke and associates (1993) by insertion of the hygromycin phosphotransferase gene (*hph*) as a selectable marker into pWFas51 (S. Oved and O. Yarden, *unpublished*). The *cna1*-antisense construct was used to transform *S. sclerotiorum* according to a standard polyethylene-glycol-mediated transformation protocol (Rollins 2003). Integration and transformant stability were verified by standard Southern analyses (Sambrook et al. 1989).

Real-time RT-PCR.

Mature sclerotia were placed on Joham's solid medium and incubated at 18ºC for 14 days. mRNA was extracted from different stages of sclerotial development: mature sclerotia, 48 h post-sclerotial germination, hyphae germinated from the sclerotia, white sclerotia (generated from the growing hyphae after approximately 6 days), and newly formed mature sclerotia reformed at the end of the experiment from the white sclerotia.

To analyze the transcript expression levels of the catalytic subunit of calcineurin, relative quantification of gene expression was performed by using SYBR Green Real-Time RT-PCR on an ABI prism 5700 sequence detection system (Applied Biosystems, Foster City, CA, U.S.A.). Two specific primers designed for each gene were used to obtain an amplicon of approximately 200 bp of each target gene (*cna1* and *tub1,* encoding the calcineurin catalytic subunit and β-tubulin control genes, respectively). The following primers were designed by utilizing PrimerExpress software (Applied Biosystems): *cna1*F-(CCTCC CACTCATGGTCTTATGTG), *cna1*R-(CGGACGTGATTGTG AATGAAGT) and *βtub*F-(TTGGATTTGCTCCTTTGACCAG), *βtub*R-(AGCGGCCATCATGTTCTTAGG). Degenerate primers previously designed for amplification of the β-tubulin gene of *B. cinerea* (Yarden and Katan 1993) were utilized for amplifying the 800-bp β-tubulin fragment, used for designing the real-time RT-PCR primers.

RT-PCR mixtures were comprised of a 12-pmol concentration of each primer, 12.5 µl of SYBR Green PCR master mix (Applied Biosystems), 10 units of Rnasin (Promega Corp.), 10 units of Expand Reverse Transcriptase (Roche, Mannheim, Germany), 15 to 20 ng of mRNA, and nuclease-free water to a final volume of 25 µl. Amplification conditions were as follows: 30 min at 48ºC, 10 min at 95°C, and then 40 cycles that consisted of 15 s at 95°C and 1 min at 60°C. Total cDNA abundance in the samples was normalized using the *tub1* gene as a control. In all experiments, samples were amplified in triplicate, and the average cycle threshold then was calculated and used to determine the relative expression of each gene. Two independent experiments were carried out in the same manner, and the final average and standard error of the relative expression values were calculated and for the two experiments analyzed by *t* test.

Calcineurin phosphatase activity.

Phosphatase activity was measured by using the calcineurin assay kit (Biomol, Plymouth Meeting, PA, U.S.A.). Total protein was extracted from the wild-type strain or CAS1 strain grown on PDA and transferred, for 24 h, to inductive (quinic acid-supplemented) or repressive (glucose-supplemented) water-agar media prior to extraction. Cell-lysate protein concentrations were quantified by the Bradford protein assay (Bradford 1976). Calcineurin activity was measured as the dephosphorylation rate of a synthetic phosphopeptide substrate (RII peptide) in the presence or absence of 10 mM EGTA (ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid). The amount of liberated PO_4 was determined colorimetrically. Results were normalized on the basis of the protein concentration in each sample. The differences in relative (quinic acid/glucose) calcineurin activity between the wild-type and CAS1 strains was analyzed by *t* test ($P < 0.05$). Each activity assay was performed in triplicate.

Pathogenicity assays.

Seed of *Arabidopsis thaliana* plants, ecotype Columbia-0, were germinated in 0.7% (wt/vol) agar plates containing MS medium (Murashige and Skoog 1962) supplemented with 1.5% (wt/vol) sucrose. Seedlings (3 weeks old) were transferred to Kekkila peat (Tuusula, Finland). Plants were cultivated for an additional 8 weeks and grown under controlled conditions: 22°C, 100 µE/m^2/s light intensity for 16 h at 70% relative humidity. Seed of tomato (*L. esculentum* Mill. cv. Bonny Best) were planted in Cocos optima (Pelemix, Moshav Katif, Israel) and grown for approximately 6 weeks in the greenhouse at temperatures ranging from 25°C (night) to 30°C (day).

Individual, fully expanded leaves of either *A. thaliana* or *L. esculentum* were excised and placed in 9-cm glass dishes lined with water-saturated No. 1 Whatman filter paper. Individual leaves were inoculated with a single 0.5-cm mycelium-colonized agar plug obtained from the expanding margins of water-medium-grown colonies and supplemented with either 15 mM quinic acid or 15 mM glucose. Inoculated leaves were maintained at 100% relative humidity at 18ºC for 96 h. The percentage of infected leaves was determined and differences between treatments analyzed by χ^2 test. Each experiment was performed with 30 leaves at least three times.

Protoplast production.

S. sclerotiorum strains were grown in PDB (Difco Laboratories) in the presence or absence of 50 nM CsA for 5 days. The harvested hyphae were washed twice with sterile distilled water and subsequently macerated (30 s) in a blender. The solution was washed three times by centrifugation (swing-out rotor) at 2,470 × *g* for 5 min and resuspended in sterile-distilled water. This treatment was repeated three times with 0.5 M sucrose. The final solution was prepared by resuspending the hyphal pellet in 4 ml of a 0.5-M sucrose solution. The hyphal suspension was incubated at 30°C at 55 rpm with NovoZyme 234 at 10 mg/ml (Novo Nordisk, Copenhagen, Denmark) to invoke protoplast production, which was monitored every 15 min by light microscopy. Results indicate the average and standard error of four replicates.

Oxalic acid quantification.

Cultures of wild-type isolate 1980 or the CAS1 strain were grown on PDA in the presence of 15 mM quinic acid or 15 mM glucose. When colonies reached the edge of the dish, the

fungal mycelium was harvested. Buffered 10 mM EDTA (pH 7.6, 8 ml) was added to the agar medium, and the mixture was heated to melt the agar. Samples were cooled to room temperature, and 3 ml was mixed with activated charcoal for 5 min. Samples were centrifuged at 1,500 × *g* for 5 min, and the supernatant was removed and diluted 10-fold in dilution buffer. The concentration of oxalic acid was determined by the Oxalate Detection Kit (Sigma-Aldrich). Oxalic acid concentration was calculated on the basis of a standard curve (using potassium oxalate) and adjusted for the dilution factors. Each experiment was performed at least twice, in duplicate. Values shown represent the mean and standard error of the experiments.

β-1,3-Glucan measurements.

The amount of β-1,3-glucan was measured using aniline blue as described previously (Shedletzky et al. 1997; Tomishige et al. 2005). *S. sclerotiorum* was cultured in PDB (control) or amended with CsA (50 nM). Glucan was extracted according to Ishibashi and associates (2004) with slight modifications. Specifically, the extracts were analyzed immediately following the NaClO treatment and acetone wash. The extracts were resuspended in 250 µl of Tris-EDTA and NaOH (6 N) was added to the cell suspension to a final concentration of 1 N. The mixture was incubated at 80ºC for 30 min, followed by the addition of 1.05 ml AB mix (0.03% aniline blue [Pharmaceutical Laboratories, New York], 0.18 N HCl, and 0.49 N glycine/NaOH, pH 9.5). The tube was vortexed briefly, then incubated at 50°C for 30 min. β-1,3-Glucan was quantified using a spectrofluorometer (Carry Eclipse, Varian, Australia). The excitation and emission wavelengths were 400 and 460 nm, respectively. β-1,3-Glucan concentration was calculated on the basis of a standard curve using laminarin (USB, Cleveland, OH, U.S.A) and normalized on the basis of protein concentration. Three independent experiments were carried out in the same manner, the final average and standard error of the normalized β-1,3-glucan concentration values were calculated, and differences between treatments were subjected to *t* test analysis.

General extracellular pectinase, cellulase, and protease assays.

PDA plugs colonized with the wild-type *S. sclerotiorum* or the *cna1*-antisense transformant were placed on pectinase, cellulase, or protease detection-medium plates supplemented with 15 mM quinic acid or 15 mM glucose, cultured at 18ºC, and further treated as described below.

For the pectinase assay, the fungus was cultured on medium containing pectin, based on Hagerman and associates (1985), with slight modifications (pectinase detection medium: pectin, 1 g/liter; maltose, 1 g/liter; KNO_3, 1 g/liter, and agarose, 15 g/liter of medium in citric acid-sodium phosphate buffer, pH 5.2). The fungi were cultured for approximately 8 to 15 days at 18ºC. Colonies were stained with fresh 0.2% (wt/vol) ruthenium red in water. The culture dishes were covered with the staining solution and refrigerated for 2 h. The stain then was discarded and the dishes destained for 15 min in distilled water. Pectinase activity was visualized as clear halos surrounding the hyphal colony.

Total secreted cellulase activity was evaluated on agar plates containing cellulase detection medium (carboxymethyl cellulose, 1 g/liter, in citric acid-sodium phosphate buffer, pH 5.2). The fungi were grown for 8 to 15 days at 18°C, after which the culture dishes were stained with 1% congo red (Sigma-Aldrich) solution for 5 min and destained several times by washing with 1 M NaCl. Orange halos on a red background indicated carboxymethyl cellulase activity (Shoseyov and Doi 1990).

Protease activity was assayed by using a gel composed of 1.5% (wt/vol) agarose and 1% (wt/vol) skim-milk powder in citric acid-sodium phosphate buffer, pH 5.2 (Poza et al. 2001). The fungus was cultured for 10 to 15 days at 18°C. Protease activity was visualized as clear halos surrounding the hyphal colony.

Infection cushion formation assay.

Mycelium-colonized agar plugs (0.5 cm in diameter) obtained from the expanding margins of PDA-medium-grown colonies supplemented with either 15 mM quinic acid or 15 mM glucose were placed onto the surface of empty petri dishes (nine per dish). The petri dishes were maintained at 100% relative humidity at 18°C for 24 h. The formation of infection cushions was monitored by light microscopy with a Zeiss Axioscope microscope.

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